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Effects of neural *androgen receptor* disruption on aggressive behavior, arginine vasopressin and galanin systems in the bed nucleus of stria terminalis and lateral septum

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ABSTRACT

In the present study, we investigated the role of the androgen receptor (AR) in the nervous system in the regulation of aggressive behavior and arginine vasopressin and galanin systems by testosterone. For this purpose, we used a conditional mouse line selectively lacking *AR* gene in the nervous system, backcrossed onto the C57BL/6J strain. Adult males were gonadectomized and supplemented with similar amounts of testosterone. When tested on two consecutive days in the resident intruder paradigm, fewer males of the mutant group exhibited aggressive behavior compared to their control littermates. In addition, a high latency to the first offensive attack was observed for the few animals that exhibited fighting behavior. This alteration was associated with a normal anogenital chemoinvestigation of intruder males. In olfactory discrimination tasks, sexual experience enhanced preference towards female-soiled bedding rather than male-soiled bedding and estrus females rather than intact males, regardless of genotype. This indicated that the behavioral alteration induced by neural *AR* mutation occurs in brain areas located downstream from the olfactory bulb. Quantification of the sexually dimorphic cell populations expressing preprovasopressin and galanin mRNAs in the bed nucleus of stria terminalis (BNST) and vasopressin-neurophysin 2 and galanin immunoreactivity in the lateral septum showed no significant differences between the two genotypes.

The present findings indicate that the neural AR is required in the expression of aggressive behavior but not in the sexual differentiation of AVP and galanin cell number in the BNST and fiber immunoreactivity in the lateral septum. They also suggest that AR in the nervous system could mediate activational effects of testosterone in the regulation of aggressive behavior during adulthood.

1. Introduction

Testosterone has a central role in the regulation of male behaviors ranging from partner preference and mating to aggression and territoriality. In male rodents, testosterone liberated by fetal and neonatal testes permanently potentiates male (masculinization) and inhibits female (defeminization) behavioral and neuroanatomical characteristics. These organizational effects of testosterone result in sex differences in brain anatomy at the structural, neurochemical and molecular levels. Sex differences in cell number and morphology or fiber density have been widely described in rat brain nuclei that regulate aggressive behavior like the bed nucleus of stria terminalis (BNST) and the medial amygdala (MeA). These brain structures contain cells expressing arginine-vasopressin (AVP), a key neuromodulator of social behaviors. Cells expressing AVP are more numerous in the male than in the female (van Leeuwen et al., 1985; De Vries and Al-Shamma, 1990). Furthermore, density of AVP fibers in the lateral septum is also more important in rat males than in females (van Leeuwen et al., 1985; De Vries and Al-Shamma, 1990). The sexual dimorphism of AVP system is organized perinatally (De Vries and Al-Shamma, 1990; Wang et al., 1993) and involves estradiol (Han and De Vries, 2003). However, it seems also maintained by activational effects of testosterone during adulthood (De Vries et al., 1994). In fact, adult castration of rats results in decreased expression of AVP mRNA and reduced AVP immunoreactivity in the BNST and the MeA, whereas testosterone supplementation restores AVP to levels noted in in-

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tact males (Brot et al., 1993; De Vries et al., 1994). In this context, both the androgenic and the estrogenic metabolites of testosterone were needed to fully restore AVP expression in the rat BNST (Brot et al., 1993; De Vries et al., 1994). In the central nervous system, testosterone acts directly by stimulating the androgen receptor (AR) or can be metabolized *in situ* by the cytochrome P450 aromatase into 17-β estradiol, which then activates its receptors ERα and ERβ. AR and ERs belong to the nuclear receptor superfamily.

The mechanisms underlying the effects of testosterone in the regulation of aggression and AVP system have been addressed by using transgenic mice ubiquitously lacking *Cyp19 aromatase* or *ERs*. Males with *Cyp19 aromatase* deletion exhibit deficient social recognition ability and reduced AVP-immunoreactivity in the BNST and MeA (Pierman et al., 2008; Plumari et al., 2002). Adult treatment of these mice with estradiol restored social recognition and AVP-immunoreactivity in the lateral septum, suggesting that the androgenic rather than the estrogenic pathway plays a role in the organizational effects of testosterone (Pierman et al., 2008). Nevertheless, data obtained in males lacking *ERα* gene showed a severe aggressive behavioral deficit (Ogawa et al., 1997) and adult treatment of these males with estradiol did not restore the behavioral deficiency (Scordalakes and Rissman, 2004). In these mice, AVP-immunoreactivity was found reduced only when both AR and ERα were lacking, suggesting that AR may contribute with ERα in the organization of AVP-immunoreactivity during development (Scordalakes and Rissman, 2004).

The direct contribution of AR in aggressive behavior has been studied in mice carrying spontaneously mutated *AR* gene (Testicular Feminization Mutation) or ubiquitously invalidated for *AR* gene (ARKO). Genetic (XY) males with Tfm or *AR* mutation have female external genitalia, undifferentiated urogenital tract and low levels of testosterone during adulthood. Aggressive behavior was found reduced in Tfm mice (Ohno et al., 1974) and ablated in ARKO animals (Sato et al., 2004). However, a comparable behavior and AVP immunoreactivity system was described in Tfm and wild type mice subjected to adult gonadectomy and estradiol supplementation (Scordalakes and Rissman, 2004).

We recently generated a conditional knockout mouse line lacking *AR* in the nervous system (Raskin et al., 2009). Unlike Tfm and ARKO mice, the conditional mutant males develop male external genitalia and functional testes producing testosterone. Only a small proportion of mutants displayed aggressive behavior (Raskin et al., 2009). However, this first study was performed on mice of mixed genetic background and did not investigate in parallel the AVP system. In fact, the effects of *AR* gene disruption on aggressive behaviors may be modified by background strain, as reported for other genes (Dominguez-Salazar et al., 2004; Le Roy et al., 2000).

The first aim of this study was the analysis of aggressive behavior in mice with a nervous system knockout of *AR* backcrossed onto C57BL/6J, a strain widely used in reproductive behavior studies (Burns-Cusato et al., 2004). As aggressive behavior is induced by olfactory cues in rodents, we investigated whether the disrupted behaviors of mutant males could be accounted for by deficiencies in olfactory preference. We also quantified mRNAs positive cells for AVP in the BNST and AVP fiber immunoreactivity in the lateral septum. Similar quantifications were performed for galanin expressing cell population known to be also sexually dimorphic in the BNST of mice (Rajendren et al., 2000).

2. Materials and methods

2.1. Animals

AR^{NesCre} mice were initially obtained in a mixed genetic background (C57BL6/J and 129SvEv), by crossing floxed AR mice and transgenic mice expressing Cre recombinase under the control of rat nestin (Nes), as previously described (Raskin et al., 2009). This mouse line was backcrossed for at least 9 generations onto the C57BL/6J strain. Mice were weaned at 24–26 days of age and housed in groups in conditions of controlled photoperiod (12 h light/12 h dark cycle – lights on at 7 am) and temperature (22 °C), with free access to food and water. Genotyping was performed as previously described (Raskin et al., 2009). All animal studies were performed in accordance with the NIH guidelines for care and use of Laboratory animals (NIH Guide) and French and European legal requirements (Decree 87-848, 86/609/ECC).

2.2. Treatments

Adult males (2–3 months of age) were anesthetized with xylazine/ketamine, gonadectomized and implanted with Silastic® tubes containing 10 mg of testosterone (Sigma–Aldrich, St.-Quentin Fallavier, France) as previously described (Raskin et al., 2012). Behavioral studies were conducted on males between two and four weeks after treatment (Groups 1 and 2). *In situ* hybridization and immunohistochemistry were performed four weeks after treatment (Group 3). In these conditions, circulating levels of testosterone are similar between control and mutant males (Raskin et al., 2012). Indeed, in the present study, the weight of the androgen-sensitive seminal vesicles was comparable between the two genotypes (0.0068 ± 0.0007% of body weight for controls *versus* 0.0064 ± 0.0004% of body weight for mutants).

Estrus C57BL/6J females (Janvier, Le Genest, France) were ovariectomized, implanted with Silastic® implants filled with estradiol-benzoate and subcutaneously treated with progesterone (Sigma–Aldrich) 4–5 h before the tests as previously reported (Raskin et al., 2009, 2012).

2.3. Resident-intruder test

Naive males of Group 1 were individually housed for two weeks without bedding changes two weeks after treatment. They were then tested in their home cages for two consecutive days by using A/J mice (The Jackson Laboratory, USA) as intruders as previously described (Raskin et al., 2009). The tests were conducted under red-light illumination 2 h after lights off and lasted 10 min. The latencies to the first anogenital chemoinvestigation and first offensive attack (defined by biting and wrestling) displayed toward the intruder mice were scored for each resident animal. When no attack occurred, the latency was 600 s.

2.4. Olfactory preference

Two weeks after treatment, naive males of Group 2 were tested for olfactory preference (Test 1). One week later, males were mated with receptive females and let to reach ejaculation as previously described (Raskin et al., 2009), before tested again one week later for their olfactory preference (Test 2). All mice exhibited a complete sexual behavior, but mutant males took longer to show the first mount, intromission and to reach ejaculation compared to their control littermates (63 ± 23 min *vs* 7 ± 1 min, 72.4 ± 24 min *vs* 10.4 ± 2 min, 94.3 ± 23 min *vs* 49.5 ± 6.3; respectively).

Olfactory preference was conducted in an enclosed Plexiglas Y-maze, under red-light illumination 2 h after lights off. Mice were initially allowed to become familiar, for 5 min on each of two consecutive days, with the experimental paradigm, but with no stimulus in the goal boxes. On the day of the test, mice were offered the choice between bedding soiled by males or estrus females and between an estrus female and a gonadally intact male. Male-soiled bedding was obtained from animals placed in a cage with clean